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Effect of Exposure to Pan and Ozone on Susceptibility to Chronic Bacterial Infection

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The effects of peroxyacetyl nitrate (PAN) and ozone (O_3) on susceptibility of mice and guinea pigs to chronic and acute respiratory infections were studied. The agent used for the acute infectious disease was Streptococcus sp. whereas Mycobacterium tuberculosis served as the agent for chronic respiratory infection. A significant increase in mortality due to streptococcal pneumonia was seen upon a single 3-hr exposure to PAN in concentrations ranging from 14.8 to 28.4 mg/m^3. Multiple daily exposures to 4.9 or 7.4 mg/m^3 PAN 3 hr/day, 5 days/week for up to 3 weeks had no effect on mortality, survival rates, or ability to clear inhaled Streptococcus sp. from the lungs. Daily 3-hr exposures to 25.0 mg/m^3 PAN did not produce any marked changes in the chronic infection as measured by M. tuberculosis titers in the lungs. The diameter of erythemas, expressing the cutaneous delayed hypersensitivity reaction were persistently smaller in guinea pigs exposed to PAN than those exposed to air. Multiple exposures to 19.8 mg/m^3 PAN resulted ininitial elevation of antibody titers, but depression of titers during the later (12 to 15 week) observation period. A single exposure to the same concentration of PAN resulted in a significant increase in total number of cells lavaged from their lungs but somewhat decreased levels of adenosine triphosphate (ATP). Exposure to 7.4 mg/m^3 PAN 3 hr/day, 5 days/week for 2 weeks resulted in reduced total cell counts and a significant reduction of ATP levels in alveolar macrophages. Scanning electron microscopic observations of the respiratory tract showed that the nonciliated cells of the nasal cavities and tracheas of mice exposed to PAN were raised and sloughing and excess mucus was present. In older mice lung congestion was enhanced by PAN exposure. Exposures to ozone resulted in increased titers of M. tuberculosis in the lungs, depression of hypersensitivity reaction and elevation in serum antibody titers.

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EFFECT OF EXPOSURE TO PAN AND OZONE ON SUSCEPTIBILITY TO CHRONIC BACTERIAL INFECTION

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report describes the results of an *in vivo* investigation designed to determine the effects of peroxyacetyl nitrate and ozone on susceptibility of mice and guinea pigs to chronic and acute respiratory infection. The agent used for the acute infectious disease was *Streptococcus pyogenes* whereas *Mycobacterium tuberculosis* served as the agent for the chronic respiratory infection. In these studies the relative comparison of the effects of these two pollutants on the resistance to laboratory induced bacterial infections of the lung could be obtained. As appropriate to the specific experiments, the parameters measured were mortality, survival time, retention or growth of inhaled microorganisms in the lung, histopathology of the respiratory tract tissue, pulmonary cellular defense system, and immunological response.

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ABSTRACT

The effects of peroxyacetyl nitrate (PAN) and ozone (O_3) on susceptibility of mice and guinea pigs to chronic and acute respiratory infections were studied. The agent used for the acute infectious disease was *Streptococcus* sp. whereas *Mycobacterium tuberculosis* served as the agent for chronic respiratory infection.

A significant increase in mortality due to streptococcal pneumonia was seen upon a single 3-hr exposure to PAN in concentrations ranging from 14.8 to 28.4 mg/m^3. The excess mortalities ranged from 8 to 39% and reduction in the survival time from 2.4 to 7.9 days. Within this concentration range of PAN a close relationship was present between the duration of exposure and concentration. Multiple daily exposures to 4.9 or 7.4 mg/m^3 PAN 3 hr/day, 5 days/week for up to 3 weeks had no effect on mortality, survival rates, or ability to clear inhaled *Streptococcus* sp. from the lungs.

Daily 3-hr exposures to 25.0 mg/m^3 PAN did not produce any marked changes in the chronic infection as measured by *M. tuberculosis* titers in their lungs. The diameter of erythemas, expressing the cutaneous delayed hypersensitivity reaction were persistently smaller in guinea pigs exposed to PAN than those exposed to air. Multiple exposures to 19.8 mg/m^3 PAN resulted in initial elevation of antibody titers, but depression of titers during the later (12 to 15 week) observation period. A single exposure to the same concentration of PAN resulted in a significant increase in total number of cells lavaged from their lungs but somewhat decreased levels of adenosine triphosphate (ATP). Exposure to 7.4 mg/m3 PAN 3 hr/day, 5 days/wee for 2 weeks resulted in reduced total cell counts and a significant reduction of ATP levels in alveolar macrophages.

Scanning electron microscopic observations of the respiratory tract showed that the nonciliated cells of the nasal cavities and tracheas of mice exposed to PAN were raised and sloughing and excess mucus was present. In older mice lung congestion was enhanced by PAN exposure.

Exposures to ozone resulted in increased titers of *M. tuberculosis* in the lungs, depression of hypersensitivity reaction and elevation in serum antibody titers.

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LIST OF ABBREVIATIONS

AM	--	alveolar macrophages
ATP	--	adenosine triphosphate
BSA	--	bovine serum albumin
CFU	--	colony-forming units
CO_2	--	carbon dioxide
CT	--	product of concentration x time
D/T	--	deaths per total number of animals
IR	--	infrared
LC_{50}	--	lethal concentration for 50% of the animals
L/min	--	liters per minute
mg/m^3	--	milligrams per cubic meter
NO_2	--	nitrogen dioxide
NO_3	--	nitrate
O_3	--	ozone
OAA	--	oleic acid albumin
PAN	--	peroxyacetyl nitrate
PBS	--	phosphate buffered saline
PPD	--	purified protein derivative
ppm	--	parts per million
RH	--	relative humidity
RMSR	--	relative mean survival rate
S.D.	--	standard deviation
S.E.	--	standard error
SEM	--	scanning electron microscopy
SO_2	--	sulfur dioxide
t_{50}	--	time required for 50% of inhaled bacteria to be cleared from the lungs
TU	--	tuberculin units
µ	--	micron

SECTION 1

INTRODUCTION

Peroxyacetyl nitrate (PAN) is the first member of a series of peroxyacetyl nitrates ($R\text{-}C(=O)\text{-}OONO_2$) that are important constituents of photochemical air pollution. PAN is a highly oxidized, unstable organic nitrogen compound that results from ultraviolet irradiation in air of certain unsaturated hydrocarbons in the presence of oxides of nitrogen. PAN was the first pure compound recognized as causing plant damage and eye irritation (1). A number of studies conducted to determine effects on animals indicate that the toxicity of PAN for mice is greater than that of sulfur dioxide (SO_2), similar to nitrogen dioxide (NO_2) and lower than that of ozone (O_3).

In studies conducted by Campbell and coworkers (2) the LC_{50} for mice for a 2-hr exposure was 105 to 150 ppm PAN. The LC_{50} was closely related to the age of the animals and the environmental temperature maintained during the exposure. Mice, 4 to 6 month old were more susceptible than those 2 to 3 month old and increased susceptibility to PAN was seen at 90°F as compared to 80°F. The same investigators reported a depression of voluntary motor activity in mice during exposure to approximately 8 ppm PAN (3). Daily 6 hr exposure to 15 ppm PAN for 6 months caused 18% mortality, weight loss, and damage to the respiratory tract tissue in mice (4). The damage included tracheobronchitis, broncholotis, pneumonitis, mild emphysema, squamous metaplasia, and epithelial hyperplasia. In a study involving 21-year-old males exposed for 5 min to 0.3 ppm PAN and then exercised for 5 min a significant increase in oxygen uptake was reported when compared to those breathing normal air (5).

Tuberculosis infection in rodents has been described by various investigators (6,7,8,9). Many of the studies have been conducted to determine the effects of vaccination on the growth of the virulent human *Mycobacterium tuberculosis* strain H37Rv in the lungs and other organs of mice and guinea pigs (6,9). The attenuated *M. tuberculosis* strain R1Rv used in our studies has been grown in liver and spleen of rats (6). It is the most virulent of the attenuated strains and causes disease in silicotic animals (Personal communications, Mr. Logy, Trudeau Institute, Saranac Lake, NY). Immunological responses, especially cutaneous delayed hypersensitivity, to *M. tuberculosis* have been measured extensively in guinea pigs (10).

Studies of the effects of inhalation of air pollutants on the resistance to chronic respiratory infection, such as tuberculosis are very limited. Thienes and coworkers (11) investigated the effects of ozone on *M. tuberculosis* infection in mice. Exposure to 1.5 ppm O_3 4 hr/day, 5 days/week for 2 months did not alter the resistance to *M. tuberculosis* H37Rv nor the BCG vaccine strain. The authors noted that this infectious model was less sensitive to ozone than the acute disease model which used *Klebsiella pneumoniae* as the infectious agent.

The overall purpose of this program was to study the effects of PAN and ozone on the susceptibility of animals to acute and chronic respiratory infections. The acute disease was established in mice by respiratory challenge with *Streptococcus* sp., (Lancefield group C) aerosols. The chronic infection was induced in mice and guinea pigs by challenge with an aerosol of *M. tuberculosis* attenuated human strain R1Rv.

The various parameters measured in mice during the chronic disease studies were the *M. tuberculosis* titers in the lungs and spleens, number of tubercules in the lungs and histopathologic changes in lungs. The parameters determined in guinea pigs were serum hemagglutination antibody titers and cutaneous delayed hypersensitivity reactions to purified protein derivative (PDD). The parameters assayed in conjunction with the acute infection included mortality and survival time, clearance rate of inhaled bacteria from the lungs, and histopathologic alterations in the lungs, tracheas, and nasal cavities as observed by light and scanning electron microscopy. In a limited number of experiments the effects of exposure to the pollutants on the pulmonary cellular defense system were determined by examining the total and differential cell counts, viability, cell surface morphology and the phagocytic function of alveolar macrophages lavaged from lungs.

SECTION 2

CONCLUSIONS

Effects of single and multiple exposures to PAN on resistance to streptococcal pneumonia were determined. Single 2 to 3 hr exposures to PAN in concentrations ranging from 14.8 to 28.4 mg/m^3 significantly enhanced the susceptibility of mice to streptococcal pneumonia. A linear relationship was observed between CT index and percent increase in mortality. The average excess mortality among mice exposed to PAN was 20%. Multiple daily 3 hr exposures to 7.4 mg/m^3 PAN for 2 weeks or 4.9 mg/m^3 for 3 weeks had no effect on mortality, survival time or the rate of clearance of inhaled bacteria from the lungs.

Studies to determine the effects of PAN on the susceptibility of mice to *M. tuberculosis* were limited by the difficulty to produce large quantities of pure PAN. The highest concentration used was 25 mg/m^3 PAN given 3 hr daily for 6 days beginning 1 week after the challenge. This exposure caused only a minimal increase in the bacterial titers in the lungs, seen in the later stages of the infection.

A 3 hr exposure of guinea pigs to 29 mg/m^3 PAN or 2.9 mg/m^3 O_3 initiated within less than 3 hr after challenge with *M. tuberculosis* aerosol indicated that both pollutants had some effect on delayed hypersensitivity. The diameters of erythema were persistently smaller in animals exposed to PAN or ozone than those exposed to air. Exposure of guinea pigs to 19.8 mg/m^3 PAN or 0.98 mg/m^3 O_3 3 hr/day for 5 days, immediately after challenge resulted in antibody titers that were generally higher in guinea pigs exposed to ozone than those exposed to air. Titers in guinea pigs exposed to PAN were initially higher but later lower than those in guinea pigs exposed to air. Exposure to 0.98 or 1.96 mg/m^3 O_3 3 hr/day 5 days/week for 6 to 8 weeks beginning 1 to 3 weeks after challenge enhanced susceptibility of mice to tuberculosis. The effect was seen as marked increases in bacterial titers in the lungs.

Other parameters measured in conjunction with the exposure to PAN included damage to lungs, tracheas and nasal cavities observed by scanning electron microscopy. The single 3-hr exposure to 25 mg/m^3 PAN caused the nonciliated epithelial cells of the nasal cavity to rise up from the basal membrane and slough. Excess mucus was also present. A similar damage was seen upon daily 3 hr exposures for 2 weeks to 7.4 mg/m^3 PAN in young, 6 week old mice. In older mice (4 month old) exposed to PAN, congestion in the lungs was greater than in air controls. Daily 3 hr exposure for 3 weeks to 4.9 mg/m^3 PAN caused damage to nonciliated cells of the trachea similar to that observed in the nasal cavities.

A single 3-hr exposure to 19.8 mg/m^3 PAN did not alter the differential cell counts or the activity and viability of alveolar macrophages. However, there was a significant increase in total cell counts (macrophages and lymphocytes). Daily 3 hr exposure for 2 weeks to 7.4 mg/m^3 PAN caused a decrease in total cell counts and reduced cellular ATP levels in alveolar macrophages.

SECTION 3

RECOMMENDATIONS

The availability of sufficiently large quantities of pure PAN is mandatory to enable the continuation of health effects studies of this pollutant. This is esepcially important for the extension of multiple and chronic exposure studies to determine their effect on the resistance to respiratory infections.

Short-term exposures to PAN alone or in combination with other pollutants such as ozone can be investigated since the effects of acute exposures to PAN were in part determined in this study. Based on results obtained, such studies should include the determination of the effects of PAN on immune response. Specifically, the activity and viability of alveolar macrophages, serum antibody formation, macrophage migration inhibition and mitogen stimulation of lymphocytes in animals exposed to PAN should be defined.

SECTION 4

MATERIALS AND METHODS

ANIMALS

Sprague-Dawley CF_1 outbred female albino mice, 6 to 16 weeks old C57BL/6 black mice 6 weeks old and Murphy Breeding Laboratory CD_2F_1 hybrid brown mice, 7 to 10 weeks old, were used in these studies. The mice were housed in groups of 10 in stainless steel shoebox cages and provided food and water *ad libitum*. Hartley strain female guinea pigs (Murphy Breeding Laboratory) weighing 300-500 g were housed in groups of 2 or 3 and provided food and water *ad libitum*.

INFECTIOUS AEROSOLS

Mycobacterium tuberculosis

Mycobacterium tuberculosis R1Rv (Trudeau Institute #205) was grown in Proskauer and Beck broth in prescription bottles incubated horizontally for 4 to 8 weeks at 37°C until confluent pellicle growth was obtained. A loopful of the pellicle was transferred to 50 ml of Middlebrook 7H9 broth (Difco) containing 0.1% Tween 80 and incubated 7 to 10 days until the culture was turbid. The unwashed culture was homogenized using a teflon pestle and 50-ml homogenizing vessel. The titer was determined by serially diluting in 0.1% phosphate-gelatin (Bacto hemagglutination buffer (Difco) with 0.1% gelatin), plating 0.1 ml aliquots onto Dubos oleic acid albumin (OAA) agar containing 50 μ/ml penicillin G and 0.1% cycloheximide, and counting the colonies after 3 weeks incubation at 37°C. The cultures were frozen in 8-ml aliquots at -70°C.

For aerosol dissemination, 6 ml of thawed culture was diluted approximately 1:10 in Middlebrook 7H9 broth to which a 10% solution of antifoam A (Dow Chemical, Midland, MI) was added. The bacterial suspension was disseminated at a rate of 0.75 ml/min from a DeVilbiss Model 841 continuous flow nebulizer using a primary filtered air flow of 8 liter/min into a 30x30x90-cm plexiglass chamber installed within a microbiological safety hood.

Groups of six guinea pigs were exposed to the aerosol for 2 min. Groups of 48 to 60 mice, housed in individual compartments of specially designed aluminum wire cages, were exposed for 20 min to the aerosol. The inhaled dose was determined by homogenizing lungs from three mice immediately after the challenge in 2 ml of 2% albumin and plating 0.2 ml of ten-fold phosphate-gelatin dilutions onto OAA agar. The plates were incubated for 4 weeks at 37°C. The number of bacteria in a total volume of 2.2 ml was considered as the number of *M. tuberculosis* per lung.

Streptococcus pyogenes

Streptococcus pyogenes group C (Lancefield strain) was passaged in mice, isolated from hearts, incubated for 18 hr at 37°C in Todd Hewitt broth and 1-ml aliquots frozen at -70°C. For aerosol dissemination, 1 ml of thawed culture was added to 100 ml of Todd Hewitt broth and incubated for 18 hr at 37°C. The culture was diluted approximately 1:10 in 0.1% peptone water and adjusted to 60% transmittance at 440 nm in a spectrophotometer (Bausch and Lomb Spectronic 20, Rochester, NY).

The bacteria were disseminated using a DeVilbiss Model 841 nebulizer by using a primary filtered air flow of 8 liter/min and a secondary humidified air flow of 28.3 liter/min into a 60x70x90-cm plexiglass chamber placed inside a microbiological safety hood.

Mice were exposed to the aerosol for 10 min. The inhaled dose was determined by homogenizing lungs from three mice in 0.1% peptone water and plating tenfold dilutions onto blood agar. The colonies were counted after 48-hr incubation at 37°C. The titer of the culture was determined by plating tenfold dilutions onto blood agar.

POLLUTANTS

Ozone (O_3)

Animals were exposed to O_3 in a 120x60x60-cm plexiglass chamber maintained at 24 $\pm$ 2°C and $\leq$40% relative humidity (RH). To prevent build-up of ammonia, deotized cage board (Upjohn Co., Kalamazoo, MI) was placed on the bottom of the chamber. A high voltage generator (IITRI) was used to convert filtered air to O_3. To provide the desired concentration, O_3 was mixed with filtered air in a glass mixing chamber and the mixture passed into the exposure chamber at a rate of 60 + 5 liters/min. Concentration was monitored continuously with an O_3 Chemiluminescent Analyzer (Model OA 310, Meloy Laboratories, Springfield, VA), and was expressed in mg/m^3 (ppm x 1.95 = mg/m^3).

Peroxyacetyl Nitrate

Synthesis--

PAN was synthesized using procedures described by Kacmarek, et al. (12). Briefly, biacetyl was photolyzed in the presence of NO_x and oxygen. Biacetyl (1-1/2 torr) and NO_2 (4 torr) were loaded into a 3 liter bulb and then oxygen was introduced. The bulb was floated in an ice bath and cold water from the bath was circulated over the bulb. The chemicals were irradiated for 4 hr by UV light from an A-H6 lamp (Illumination Industries, Sunnyvale, CA) placed 35 cm from the bulb. Over 5 ml of the gas (equivalent at STP) produced from this photolysis yielded 80%.

$$(CH_3C(O)C(O)CH_3 + NO_2 \xrightarrow[h\nu]{O_2} CH_3C(O)OONO_2)$$

Crude PAN was initially purified by vacuum line fractionation to remove methyl nitrate, carbondioxide, and excess NO_2, followed by gas chromatographic purification. The 152 cm, 0.64 cm O.D. pyrex tube column contained Fluoropak 80 support with a KEL-F grease (10%) stationary phase. The purity of the PAN was determined by IR analysis. PAN was allowed to decompose in a pyrex infrared cell at 10 torr pressure. The decomposition products in order of decreasing quantities were: CO_2, CH_3OHO_2, CH_2NO_2, CO, O_2 and CH_4. The half life of PAN under these conditions was 100 hr.

Exposure to PAN--

Pure PAN was introduced into a 60x70x40 cm plexiglass exposure chamber by passing N_2 at a rate of 300-700 ml/min over liquid PAN held at -30°C in Freon 12 in a modified cold trap. A speedaire filter and hydropurge removed moisture and oil from the nitrogen line. The chamber temperature was maintained at 26.5° ± 1.0°C. The relative concentration of PAN in the chamber was monitored continuously using a Mast oxidant analyzer containing KI solution, with an attached strip chart recorder (Model 725-3CS Mast Development Co., Davenport, IA). The concentrations were maintained by adjusting N_2 and air flow rates. All PAN concentrations (ppm x 4.95 = mg/m^3) were based on an NO_x Chemiluminescent Analyzer (Model 7101-B, Bendix Corp., Ronceverte, WV), which was used to calibrate the Mast Analyzer.

To confirm the chemiluminescent measurements, the concentration and purity of PAN in the chamber were analyzed in an infrared spectrophotometer (Perkin-Elmer Model 283) equipped with a wave number marker and a repetitive scan device. PAN was monitored at 1165 cm^{-1} and methyl nitrate at 1019 cm^{-1}. Samples were passed into a glass cell with CsI windows having a pathlength of 11 cm using inlet and outlet ports. A calibration spectrum was obtained by bubbling argon at 200 to 700 ml/min through PAN contained in the cold trap held at -30°C and passing the saturated gas through the cell. The efficiency of delivery of PAN from the vessel into the IR cell was calculated by measuring the vapor pressure of the existing PAN and comparing it to the vapor pressure of the saturated gas.

To determine PAN concentration in the animal exposure chamber a slight positive pressure was maintained in the chamber and exhausted through the IR cell. After a large volume of gas (compared to the cell volume) passed through the cell, the cell was valved-off and placed into the spectrophotometer. The concentration of PAN determined by IR was compared to that measured by the Mast Analyzer-Chemiluminescent Analyzer method.

The concentrations of PAN in the exposure chamber determined by the IR analysis were in the range of concentrations of NO_x determined by the Mast Analyzer-NO_x Chemiluminescent Analyzer measurements. Based on the qualitiatve and quantitative IR analyses it was concluded that:

- The PAN was ≥90% pure.
- The gaseous PAN did not contain break-down products, such as, methyl nitrate.
- The efficiency of generation of gaseous PAN from the liquid state into the chamber was ≥60%.
- The concentration of PAN in the chamber determined with 50% accuracy by IR was within the range determined by chemiluminescent analysis (4.5 ppm versus 3.0 ppm).
- Methyl nitrate was detected when animals were present in the chamber, suggesting that breakdown of PAN upon contact with animals, cages, and in the presence of exhaled CO_2 is inevitable. While breakdown products could have contributed to NO_x levels assumed to be PAN by chemiluminescent and oxidant analysis, the flow rates of N_2 and air required to maintain the desired PAN concentrations in the chamber were similar with and without animals. Moreover, the IR analysis specific for PAN indicated higher rather than lower concentrations compared to chemiluminescent measurements.

PARAMETERS MEASURED

Chronic Infection

Tubercles on Lungs--

At weekly intervals lungs were removed from five mice per group and placed in buffered formaldehyde. Tubercles present on the surface were counted and average counts in pollutant-exposed and control mice were compared.

Lung Tissue Histology--

Lungs from three mice per group were cannulated and perfused with buffered formaldehyde. Tissues were processed for light microscopy by conventional histologic methods and sections were cut at 5μ and stained with hematoxylin and eosin.

M. tuberculosis Titers in Lung and Spleen--

M. tuberculosis present in the lungs and/or spleens was determined at weekly intervals for 8 weeks. At each interval 5 mice were killed and the lung or spleen tissue homogenized in 2 ml of 2% albumin. Approximate tenfold dilutions were made in 0.1% phosphate-gelatin and 0.2 ml aliquots from two dilutions were plated in triplicate onto OAA agar using an Oxford Sampler micropipettor (Fisher). Thirty plates were sealed in a CO_2-impermeable 29x46 cm supervac bag (VacPac Mfg. Co., Balitmore, MD) and incubated at 37°C. Plates containing 1 to 350 colonies were counted after 3 and 4 weeks incubation. The

colony-forming units (CFU) from all plates were averaged and multiplied by the dilution factor (11) to give the total *M. tuberculosis* in 2.2 ml of homogenized tissue suspension.

The $\log_{10}$ of the CFU from each lung or spleen was averaged for each time period and the concentrations of *M. tuberculosis* in the experimental groups were compared with those in the controls using the Student t test.

Skin Sensitivity--

At weekly intervals, guinea pigs infected with *M. tuberculosis* were given a 0.1 ml (25 TU) intradermal injection of a 5.0 µg/ml buffered solution of tuberculin PPD (Parke, Davis and Co., Detroit, MI or Connaught Labs., Willowdal, Ontario, Canada) and 24 hr later the diameter of erythema was measured (mm). The difference of diameters between polutant-exposed and control animals were compared by Student t test.

Passive Hemagglutination Test--

Guinea pigs were bled weekly by cardiac puncture, approximately 1.5 ml of blood was drawn and the sera stored at -70°C. Before use, the sera were heat-inactivated at 56°C for 30 min and diluted 1:2 in isotonic saline. The procedures for tanning and sensitizing the red cells were those of Boyden (13) and Middlebrook and Dubos (14). The tests were performed using modifications of the Indirect Hemagglutination Test (Conrath (15) and the method of Daniel (16).

Briefly, citrated sheep red blood cells (Colorado Serum Co., Denver, CO) were washed three times in isotonic saline and packed by centrifuging at 2000 rpm for 5 min. A 5% red cell suspension in buffered saline at pH 7.2 was treated with a 1:40,000 (wt/vol) tannic acid solution for 10 min in a 37°C water bath. The cells were then washed twice and resuspended to half the original volume in buffered saline (PBS) providing a 10% red cell suspension. One half of the tanned cells were sensitized by adding one volume of a PPD solution (2.5 mg/ml in PBS) and incubated in a 37°C water bath for 15 min. The cells were washed in 0.1% PBS and resuspended to twice the original volume (2.5%) in the same diluent. Each serum was tested with both sensitized and unsensitized cells to assure absence of nonspecific agglutination.

Sera were adsorbed twice to the sheep red cells to remove nonspecific agglutination factors. One-tenth volume of packed, washed red cells was added to one volume of serum. After frequent shaking at room temperature for 20 min the suspension was centrifuged, the supernatant re oved, and treated again in the same manner. The tests were performed in microtiter disposotrays (V) (Linbro Scientific Co., Hamden, CT). A 0.025 ml volume of 0.1% BSA was added to all wells except the first ones where 0.05 ml of the 1:2 dilution of each serum was added. Twofold dilutions were made using 0.025 ml microdiluters. A second 0.025 ml volume of 0.1% BSA was added to each well.

To the first row of every serum sample, 0.05 ml of 2.5% tanned, unsensitized red cells was added; to the second row, 0.05 ml of 2.5% tanned, sensitized red cells was added. A saline control was used for each type of cells. The plates were incubated in a 37°C water bath for 45 min, the results read, and reread after overnight storage at 4°C. The reciprocal of the highest dilution causing ≥50% specific hemagglutination was considered the end point. Because of nonspecific agglutination of normal guinea pig serum to sheep erythrocytes, prevaccination serum for each guinea pig was tested along with each postvaccination serum.

Acute Infection

Mortality and Mean Survival Rate--

During the experiments with *S. pyogenes* the mice were observed over a 28-day period after the infectious challenge. The percent mortality and relative mean survival rate (RMSR) were determined. The RMSR were calculated according to the following equation:

$$RMSR = \frac{(AxB)+(dxL)}{n},$$

where A is the last day on which any individual mouse was alive; B is the number of mice surviving A days; d is the last day of observation; L is the number of mice alive on day d; and, n is the original number of mice in the experimental group.

The significance of the observed differences in mortality rates was determined by the chi-square test with a 2x2 contingency table. Analysis of variance, the Student t test and linear regression analysis were used when appropriate.

Bacterial Clearance Rates from Lungs--

The clearance rate of inhaled viable *S. pyogenes* from the lungs was determined by sacrificing groups of 4 mice at 0,1,2,3 and 4 hr after challenge, aseptically removing, weighing and homogenizing the lungs in 2.0 ml peptone water, and plating 0.1 ml aliquots in duplicate onto blood agar plates. The colonies were counted after 48 hr incubation at 37°C. The number of *S. pyogenes* per gram of lung was determined for each exposure condition at 0 time and this number was designated as 100% recovery. At each time interval the number of *S. pyogenes* per gram of lung was expressed as percent of the 0 hr. The rate of clearance, i.e., the time required for 50% of the original bacterial population to be cleared from the lungs (t_{50}), was determined on a semilogarithmic regression after converting the percent recoveries to $\log_{10}$ values. The slopes of the regression lines for the experimental and control groups were compared using the Student t test.

Pulmonary Cell Population--

The free cells in the lungs were obtained by tracheobronchial lavage. Mice were killed by intraperitoneal injection of sodium pentobarbital and the lung-heart-trachea complex removed *in toto*. A blunted hypodermic needle inserted into the trachea and fastened with surgical suture was used to lavage the lungs by repeated infusions of warm saline.

The cells were isolated from the lavage fluids by centrifuging at 365 xg and washing in Hanks' medium. Total cell counts were made using a white blood cell diluting pipette and a hemocytometer. To determine the cellular distribution (i.e., the proportion of alveolar macrophage, polymorphonuclear leukocytes and lymphocytes), differential counts were made on air-dried smears of cells fixed in methanol and stained with Wright's stain. Viability of alveolar macrophages (AM) was determined by dye exclusion using 1% trypan blue. At least 200 cells selected at random were counted microscopically for determination of differential counts and viability.

Cellular adenosine triphosphate (ATP) concentration in the lavaged cells was determined using a DuPont 760 Luminescence Biometer employing procedures recommended for the instrument. The assay is based on the principal that when a microsample containing ATP is injected into a suitably buffered reaction mixture of luciferase and luciferin, the peak intensity of the resulting light flash is directly proportional to the concentration of ATP. ATP was extracted from the cells by dimethyl sulfoxide (DMSO) from aliquots of the lavaged and washed cellular suspension. The DMSO extracts were diluted with 0.01 M morpholinopropane sulfonic acid (MOPS) buffer to overcome the quench effect of the high concentration of DMSO in the aqueous extract. ATP levels were expressed as femtograms (10^{-15}g) of ATP per 10^5AM (calculated from initial cell counts).

In vitro phagocytic activity was evaluated by light microscopy. Macrophages obtained from individual mice were resuspended to provide $4x10^5$AM/ml in Medium 199 in Hanks' salt solution supplemented with Hepes buffer, Gentamicin (Schering Corp.) and heat-inactivated fetal calf serum. A saline suspension of polystyrene latex particles (1.1μ) was prepared to contain $2x10^8$ particles/ml. To determine the phagocytic activity 0.5 ml aliquots of AM suspension were mixed with 0.1 ml of the particle suspension resulting in a final concentration of $3.33x10^7$ particles and $3.33x10^5$ AM/ml, (100 to 1 ratio of particles to AM). After 1 hr incubation at 37°C the AM were washed in Hanks' salt solution, centrifuged at 365 xg for 5 min, smeared onto glass slides, fixed in formalin vapor overnight and stained with methylene blue. The non-engulfed latex spheres were removed from the slides by a 3-hr immersion in xylene. The percentage of phagocytizing cells was determined for each lavage sample by counting 200 cells on duplicate slides.

For scanning electron microscopy examination isolated AM were resuspended in Hanks' medium supplemented with heat-inactivated fetal calf serum and Gentamicin. One-ml aliquots of this suspension containing between 3.5 and $7.5x10^5$ were incubated at 37°C in a 5% CO_2 humidified atmosphere for 1 3/4 hr in plastic petri dishes containing glass coverslips as substrates to allow the cells to attach. Subsequently the coverslips were removed, rinsed with warm PBS and placed into 1% glutaraldehyde fixative-solution prepared in PBS. After fixation, the coverslips with the attached cells were washed in a gentle stream of distilled water, immediately frozen by immersion into liquid Freon-22, and freeze-dried in vacuum.

For scanning electron microscopy (SEM) examination the coverslips were glued to metal specimen holders. To render them conductive, the macrophages were covered with a thin layer of carbon followed by gold evaporated onto their surface in high vacuum. Micrographs were taken at 20 kV in a Kent-Cambridge Mark II Stereoscan scanning electron microscope.

Scanning Electron Microscopy--

Tissues from the nasal cavities, tracheas, and lungs were taken for SEM examination from mice exposed to PAN or filtered air and challenged $\leq$1 hr later with *S. pyogenes*. The mice were anesthetized by an intraperitoneal injection of pentobarbital, exsanguinated from the ventral aorta, the chest opened, and the lungs and trachea were removed *in toto*. The trachea was cannulated to the level of the first cartilaginous ring and the lungs expanded with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative at 20 cm water pressure. Perfusion continued for at least 2 hr with the lungs completely immersed in fixative. Upon completion of airway perfusion, the trachea was ligated and the lungs floated in fixative.

After cannulation of the trachea, the head was removed, the skin was retracted and the lower jaw removed by sectioning through the ramus. The cranium was cut off immediately posterior to the orbits, leaving only the nasal cavity. The nasal bones were reflected utilizing small forceps and the cavity halved by inserting a razor blade on one side of the medium septum and severing the hard palate.

The trachea and main stem bronchi were isolated from the lungs. The lungs were sectioned with a razor blade in order to reveal the bronchus and alveoli of each lobe. All tissues were washed in distilled water and dehydrated with increasing concentrations of alcohol. Pentyl acetate was then substituted for the alcohol and the tissues were dried by the critical point method in carbon dioxide. The dried trachea was sectioned longitudinally. The tissues were cemented to stubs, gold-coated in a Denton vacuum evaporator equipped with a rotating turntable, and examined in a Kent-Cambridge Mark II Stereoscan scanning electron microscope at 20kV.

Samples of lung, trachea and nasal cavities were also processed for light microscopy examination. Sections were cut at 5μ and stained with hematoxylin and eosin.

SECTION 5

RESULTS AND DISCUSSION

HEALTH EFFECTS OF EXPOSURE TO PAN

The effects of PAN on the resistance to acute and chronic respiratory infections were studied in mice and guinea pigs. In some of the acute infection experiments, initiated by inhalation of *Streptococcus* sp. aerosol, mice exposed to ozone were included in parallel with those exposed to PAN. Thus relative comparison of the effects of these two pollutants on the resistance to streptococcal pneumonia could be obtained. As appropriate to the specific experiments the parameters measured were mortality, survival time, retention or growth of inhaled bacteria in the lungs, histopathology of respiratory tract tissues, pulmonary cellular defense system, and immunological response.

Effect on Resistance to Streptococcal Pneumonia

Mortality and Survival Rates--

Mortality rates and mean survival time of mice resulting from single 2 or 3 hr exposures to various concentrations of PAN and challenged within 1 hr with *Streptococcus* sp. aerosol are shown in Table 1. Significant increases in mortality and decreases in mean survival time were seen in mice exposed to PAN in concentrations ranging from 14.8 to 28.4 mg/m^3. The mean mortality rate for the six exposure conditions was 31.7% ($\pm$7.5% S.E.) and excess mortality ranged from 8 to 39%. The reduction in the survival time ranged from 2.4 to 7.9 days. The mean mortality of mice exposed for 2 or 3 hr to 0.98 mg/m^3 O_3 and challenged with the infectious aerosol, which were included in the experiments as positive controls, was 37.9% ($\pm$6.5% S.E.).

To further assess the effects of the short term exposure to PAN the CT index, representing the products of PAN concentration and the duration of exposure was calculated. The percent changes in mortality observed at each exposure condition were subjected to regression analysis and the resulting least square lines were plotted against the CT index (Figure 1). The relationship between excess mortality due to streptococcal pneumonia and the CT index can be clearly seen (correlation coefficient $r = 0.95$, $p \leq 0.01$).

TABLE 1. MORTALITY AND SURVIVAL RATES OF MICE EXPOSED FOR 2 OR 3 HR TO PAN AND CHALLENGED WITHIN 1 HR WITH *STREPTOCOCCUS* AEROSOL

PAN Exposure		Mortality					RMSR (day)		
Concn	Time	Air		PAN		Change			
mg/m^3	hr	D/T	%	D/T	%	%	Air	PAN	Change
14.8	3	6/90	7	14/91	15	+ 8**	26.5	24.1	-2.4
19.8	3	4/147	3	23/142	16	+13*	27.4	24.6	-2.8*
22.3	2	6/49	12	13/50	26	+14**	26.0	22.5	-3.5*
24.7	3	40/150	27	84/150	56	+29*	22.3	14.7	-7.6*
27.2	2	9/100	9	23/100	23	+14*	27.2	23.0	-4.2*
28.4	3	30/201	15	105/196	54	+39*	23.2	15.3	-7.9*

Significant change from corresponding infected mice exposed to filtered air.

* $p \leq 0.05$.

** $p \leq 0.1$.

Multiple daily exposures to PAN did not affect the resistance of mice to the streptococcal infection. Daily exposure 3 hr/day, 5 days/week to 4.9 mg/m^3 PAN for 2 and 3 weeks (10 and 15 exposures) or to 7.4 mg/m^3 for 2 weeks (10 exposures) had no appreciable effect on mortality or mean survival time (Table 2).

TABLE 2. MORTALITY AND SURVIVAL RATES OF MICE DAILY EXPOSED 3 HR/DAY 5 DAYS/WEEK FOR 2 OR 3 WEEKS TO PAN AND CHALLENGED WITHIN 1 HR WITH *STREPTOCOCCUS* AEROSOL

PAN Exposure		Mortality					RMSR (days)		
Concn	Duration	Air		PAN		Change			
mg/m^3	(days)	D/T	%	D/T	%	%	Air	PAN	Change
4.9	10	31/160	19	41/159	26	+7	23.8	22.6	-1.2
4.9	15	2/30	7	2/27	8	+1	26.6	26.4	-0.2
7.4	10	17/75	23	12/77	16	-7	23.2	25.0	+1.8

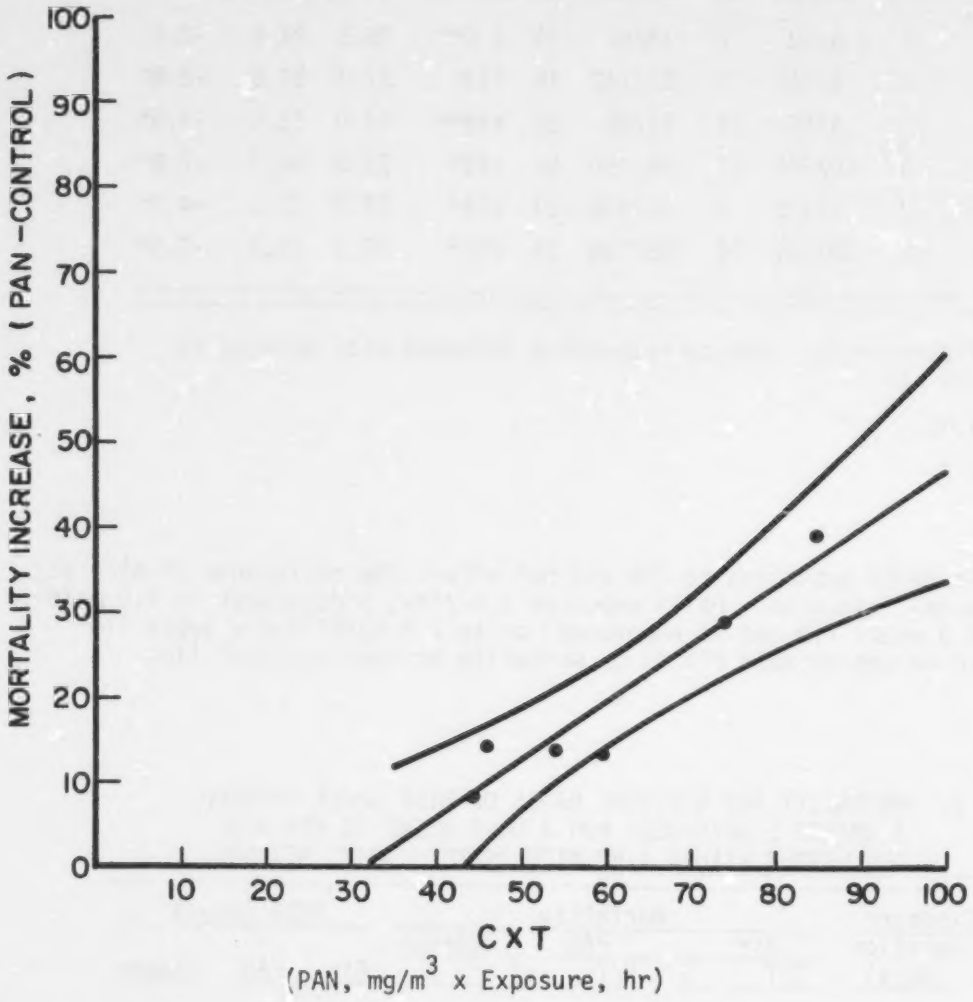

Figure 1. Relationship between excess mortality due to streptococcal pneumonia in mice and CT index for PAN exposure (Mean and 95% CL).

Lung Clearance of Inhaled Bacteria--

The rates of clearance of inhaled viable *Streptococcus* sp. (t_{50}) from lungs of mice after single and multiple 3-hr exposures to PAN were not significantly different from those seen in mice exposed to air (Table 3). After single 3-hr exposures to 14.8 or 19.8 mg/m^3 PAN clearance rates fluctuated with respect to air controls, although mortality rates were significantly higher in mice exposed to the same concentrations of PAN. Upon repeated daily exposures 3 hr/day, 5 days/week for 2 or 3 weeks to 4.9 or 7.4 mg/m^3 PAN the clearance rates were similar to those seen in mice exposed to air.

TABLE 3. CLEARANCE OF INHALED *STREPTOCOCCUS* FROM LUNGS OF MICE EXPOSED TO PAN

PAN Exposure		Mice		Clearance Rate t_{50} (hr)[a]	
Concn mg/m^3	Number of 3-hr Exposures	Strain	Age (wks)	Air	PAN
14.8	1	CDF_1	9-12	1.13	1.00
19.8	1	CDF_1	5	0.66	0.84
4.9	10	CF_1	16	1.10	1.25
7.4	10	CF_1	6-9	0.96	1.09
4.9	15	CF_1	8	1.20	1.00

[a] Time in hours required to clear 50% of inhaled viable bacteria.

Effect on Resistance to Tuberculosis

To study the effects on the resistance to chronic mycobacterial infection mice and guinea pigs were exposed to various concentrations of PAN. Inasmuch as inhalation of *M. tuberculosis* aerosols per se did not result in mortalities, other indicators of changes in the resistance to the infection were used. Initially these parameters included the determination of the numbers of tubercles present on the lung surface and histopathological changes in the respiratory tract.

Neither parameter provided satisfactory information as to the course or the severity of the infection. Formation of tubercles on the lung surface did not occur before the 4th week and the tubercles were difficult to count. Typical focal granulomas appeared at 3 weeks and became confluent during the later stages of the infection so that the differences between lungs were difficult to quantitate.

Mycobacterial Titers in Lungs--

The changes in resistance to the infection, as determined by *M. tuberculosis* titers in lungs were studied in mice exposed to PAN and compared to those in mice exposed to filtered air. In all experiments the mice were challenged first with *M. tuberculosis* aerosol and exposed to various PAN regimens after the infectious challenge.

The inhaled dose of *M. tuberculosis* ranged from 45 to 125 CFU/lung and there was no apparent increase in the titers at 1 week after the infectious challenge. In control mice exposed to filtered air the titer of mycobacteria showed a continuous increase reaching a peak concentration at 4 to 5 weeks after the infectious challenge. In general the titers remained approximately constant for an additional 2 to 3 weeks and showed a slow decrease thereafter.

Daily 3 hr exposure to 25.0 mg/m^3 PAN for 6 days beginning one week after the respiratory challenge with *M. tuberculosis* aerosol did not produce any changes in the infection in mice, as measured by bacterial lung titers. In mice exposed to PAN and those exposed to filtered air the titer of *M. tuberculosis* in lungs reached a peak at approximately 4 weeks after the infectious challenge and a slight decrease during an additional 6 week holding period was seen.

Immune Response--

The cutaneous sensitivity to PPD was determined in guinea pigs challenged with *M. tuberculosis* aerosol and within less than 3 hr exposed for 3 hr to 29 mg/m^3 PAN or exposed daily 3 hr/day for 5 days to 19.8 mg/m^3 PAN. After the single 3-hr exposure to 29 mg/m^3 PAN the diameter of erythema increased gradually until a peak response was seen at 6 weeks after the infectious challenge. Between the 7th and 18th week after the challenge a slow decrease of the erythema was seen. Guinea pigs exposed to filtered air responded in a similar manner. After five daily 3-hr exposures to 19.8 mg/m^3 PAN a very slow increase in diameter of erythema was seen and a peak occurred at 11 weeks. In guinea pigs exposed to filtered air a more rapid increase in diameter was seen whereas the peak diameter was reached at 8 weeks after the infectious challenge. In general during the 16 weeks observation period the diameters of erythemas, expressing the cutaneous delayed hypersensitivity reaction were persistently smaller in guinea pigs exposed to PAN than those exposed to air. The differences in diameter ranged from approximately 0.5 to 2.0 mm, however because of the wide variations in responses of individual guinea pigs the statistical significance of the differences could not be ascertained.

The serum hemagglutination antibody titers were determined at approximately 3-week intervals in guinea pigs exposed to 19.8 mg/m^3 PAN 3 hr/day for 5 days. The multiple exposures resulted in elevated antibody titers at 3, 6 and 9 weeks after the challenge but lower titers at 12 and 15 weeks when compared to those in guinea pigs exposed to filtered air (Figure 2). At 26 weeks after the challenge the serum antibody titers were identical in both groups of animals.

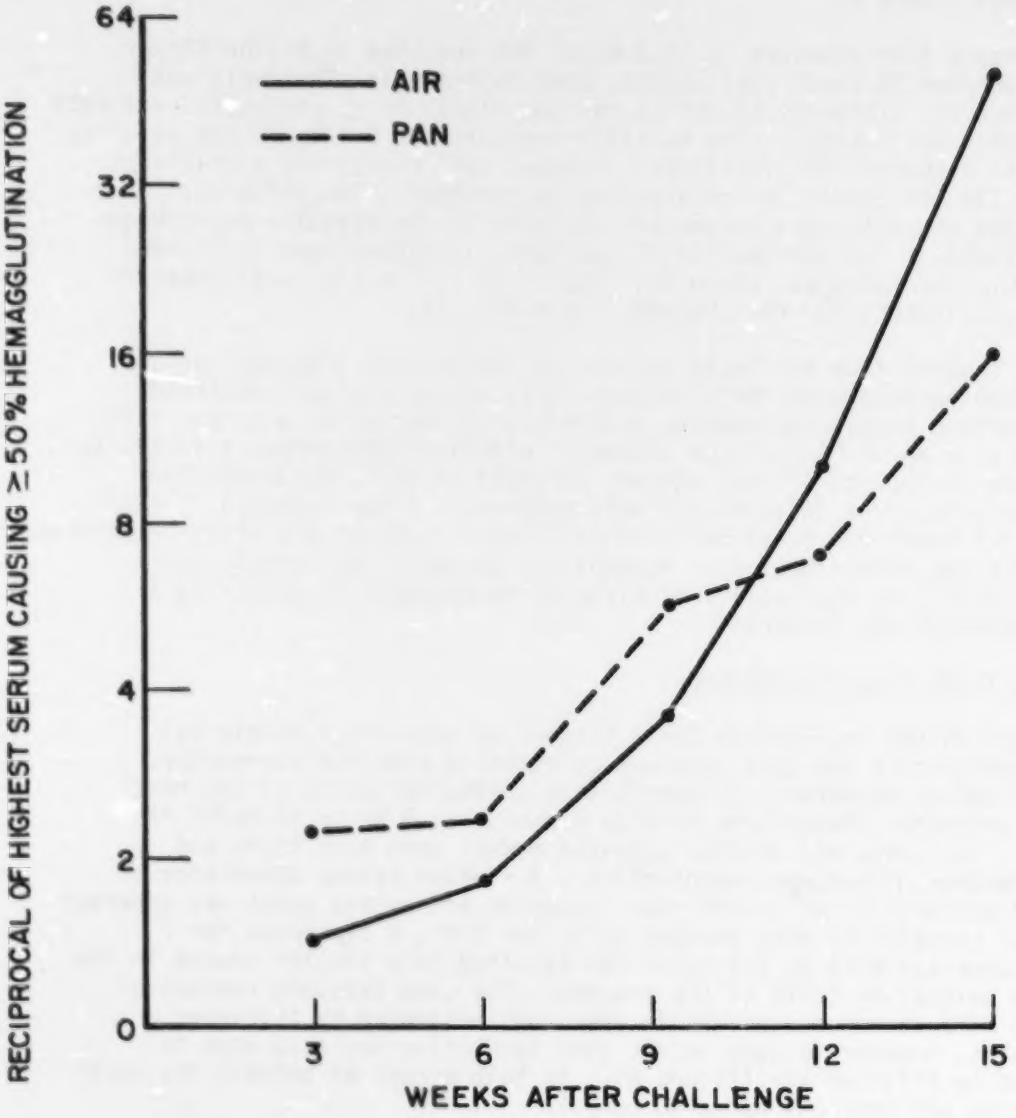

Figure 2. Serum hemagglutination antibody titers in guinea pigs challenged with *M. tuberculosis* and within <3 hr exposed to 19.8 mg/m^3 PAN 3 hr/day for 5 days.

Effects on Free Pulmonary Cells

Limited experiments were conducted to determine the effect of exposure to PAN on free cells lavaged from lungs of mice. The treatment groups included mice exposed for 3 hr to 19.8 mg/m^3 PAN and those exposed daily, 3 hr/day, 5 days/week for 2 weeks to 7.4 mg/m^3 PAN. Mice maintained in filtered air for corresponding periods of time were included with each exposure regimen (Table 4).

The single 3-hr exposure to 19.8 mg/m^3 PAN resulted in a significant ($p \leq 0.05$) increase in total cell counts, some decrease in ATP levels but did not affect the differential counts nor the viability or phagocytic activity of the alveolar macrophages. The multiple exposures to 7.4 mg/m^3 PAN resulted in a decrease although not significant in total cell counts and significant decrease in the ATP levels in the alveolar macrophages. The differential counts and the viability and phagocytic activity of the alveolar macrophages were not affected. The decrease in ATP activity indicates some impairment to the alveolar macrophages, since utilization of ATP in the cells provides the energy requirement for the phagocytic process (17).

Cells lavaged from the lungs of mice in the various treatment groups were incubated to determine their ability to attach to a glass substrate. Scanning electron microscopy examination indicated that after a single e-hr exposure to 19.8 mg/m^3 PAN a large number of alveolar macrophages attached to the glass; no surface structural changes compared to cells obtained from control mice exposed to filtered air were apparent. After repeated 10 exposures to 7.4 mg/m^3 PAN practically no cells were found on the glass substrates and therefore the determination of morphologic changes could not be made. Thus, it appears that the ability of alveolar macrophages to attach to a suitable substrate was impaired.

Respiratory Tract Tissue Morphology

Changes in the respiratory tract tissues of mice after single and multiple exposures to PAN were examined by scanning electron microscopy. An abnormal raised appearance of nonciliated epithelial cells of the nasal cavity was the major change seen in mice exposed for 3 hr to 25 mg/m^3 PAN (Figure 3). The lungs and trachea appeared normal upon both light and scanning electron microscopic examination. A similar raised appearance of nonciliated epithelial cells with some sloughing and excess mucus was apparent in the nasal cavities of mice exposed daily for 3 hr, 5 days/week for 2 weeks of 5-week-old mice to 7.4 mg/m^3 PAN resulted in a similar damage in the nonciliated epithelial cells of the trachea. The same exposure regimen of 16-week-old mice caused congestion in the lungs evidenced by thickened alveolar walls, however to some extent this thickening was also seen in mice exposed to filtered air (Figure 6). In both groups of animals the nasal cavity tissues and tracheas appeared normal.

TABLE 4. EFFECT OF EXPOSURE TO PAN ON FREE CELLS LAVAGED FROM THE LUNGS OF MICE[a]

Exposure		Cell Count								Alveolar Macrophage					
Concn mg/m^3	Number of 3 hr Exposures	Total x10^5		Differential						Viability, %		Phagocytosis,[b] %		ATP	
				Macrophage		Lymphocytes		Neutrophils							
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	1	6.92	0.72	97.0	1.5	3.0	1.5	<1		95.2	1.3	91.0	2.0	1.03	0.05
19.8	1	9.95*	1.03	97.7	0.9	2.3	0.9	<1		96.7	0.5	88.0	1.0	0.89	0.05
0	10	8.86	0.86	97.7	0.7	2.3	0.7	<1		95.2	0.9	89.9	1.8	0.98	0.05
7.4	10	6.11	1.10	96.0	1.2	4.0	1.2	<1		95.6	0.5	90.6	1.1	0.81*	0.05

[a] Cells lavaged from 6 mice, except for 4 mice used for total cell count after 10 exposures to PAN.

[b] Percent of alveolar macrophages engulfing at least one latex sphere after 1 hr incubation at 37^{o}C.

* Significant change from corresponding mice exposed to air ($p \leq 0.05$).

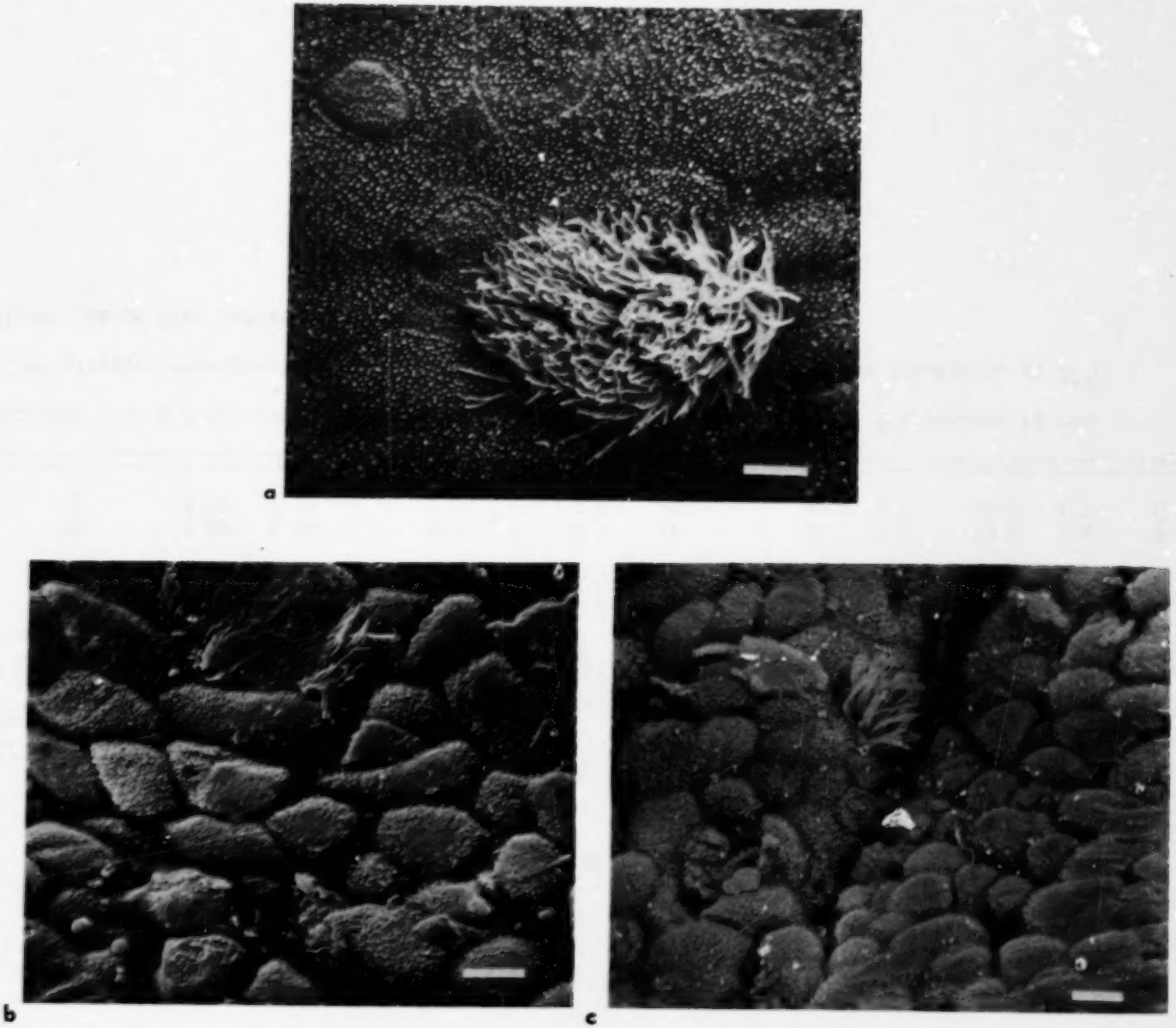

Figure 3. Nonciliated epithelial cells in nasal cavities of mice. (a) normal; (b) and (c) raised and clumped cells in mice exposed for 3 hr to 25 mg/m^3 PAN (5 μm).

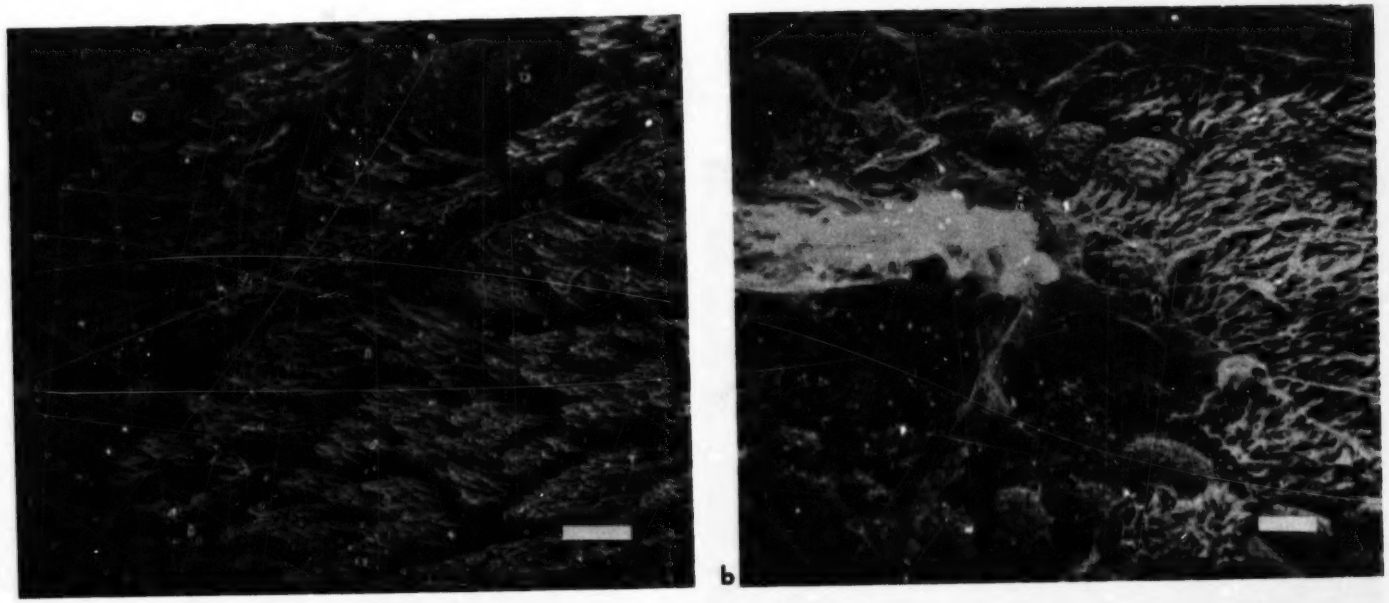

Figure 4. Ciliated epithelial cells in nasal cavities of mice. (a) normal; (b) excess mucus in mice exposed to 4.9 mg/m^3 PAN, 3 hr/day, 5 days/week for 3 weeks (5 μm).

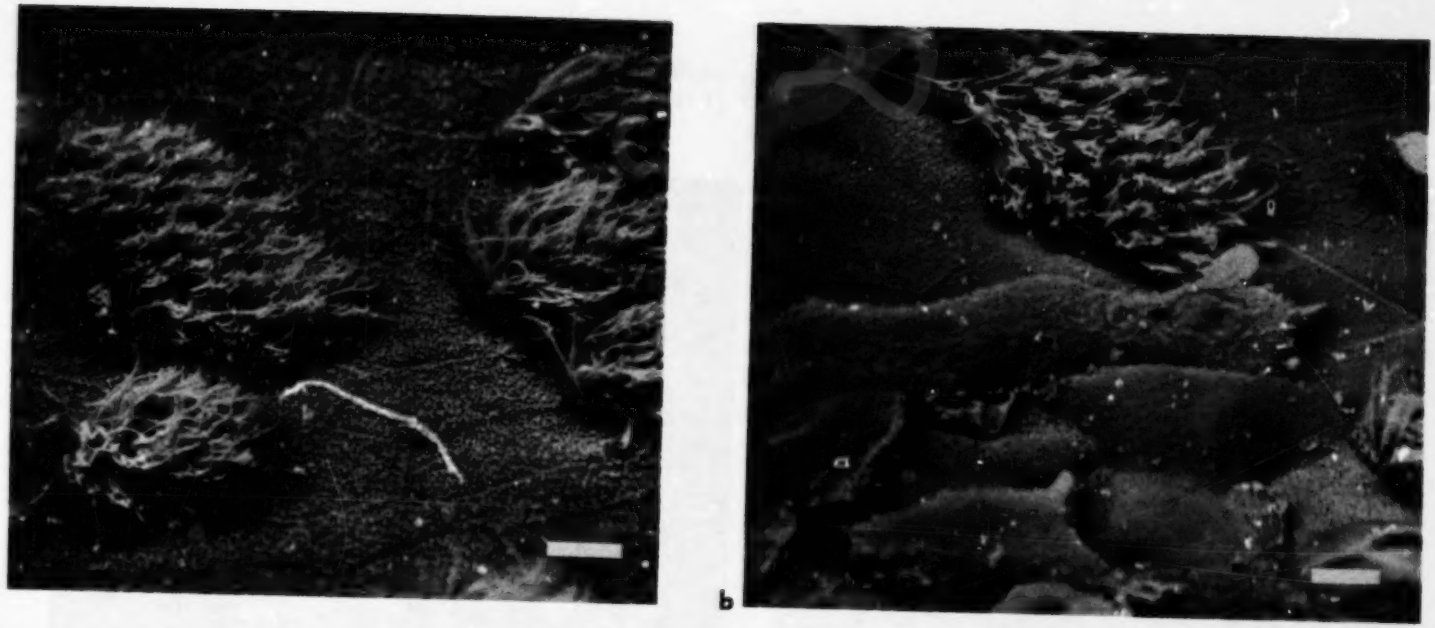

Figure 5. Nonciliated epithelial cells in nasal cavities of mice. (a) normal; (b) raised and sloughing cells in mice exposed to 4.9 mg/m^3 PAN, 3 hr/day, 5 days/week for 3 weeks (5 μm).

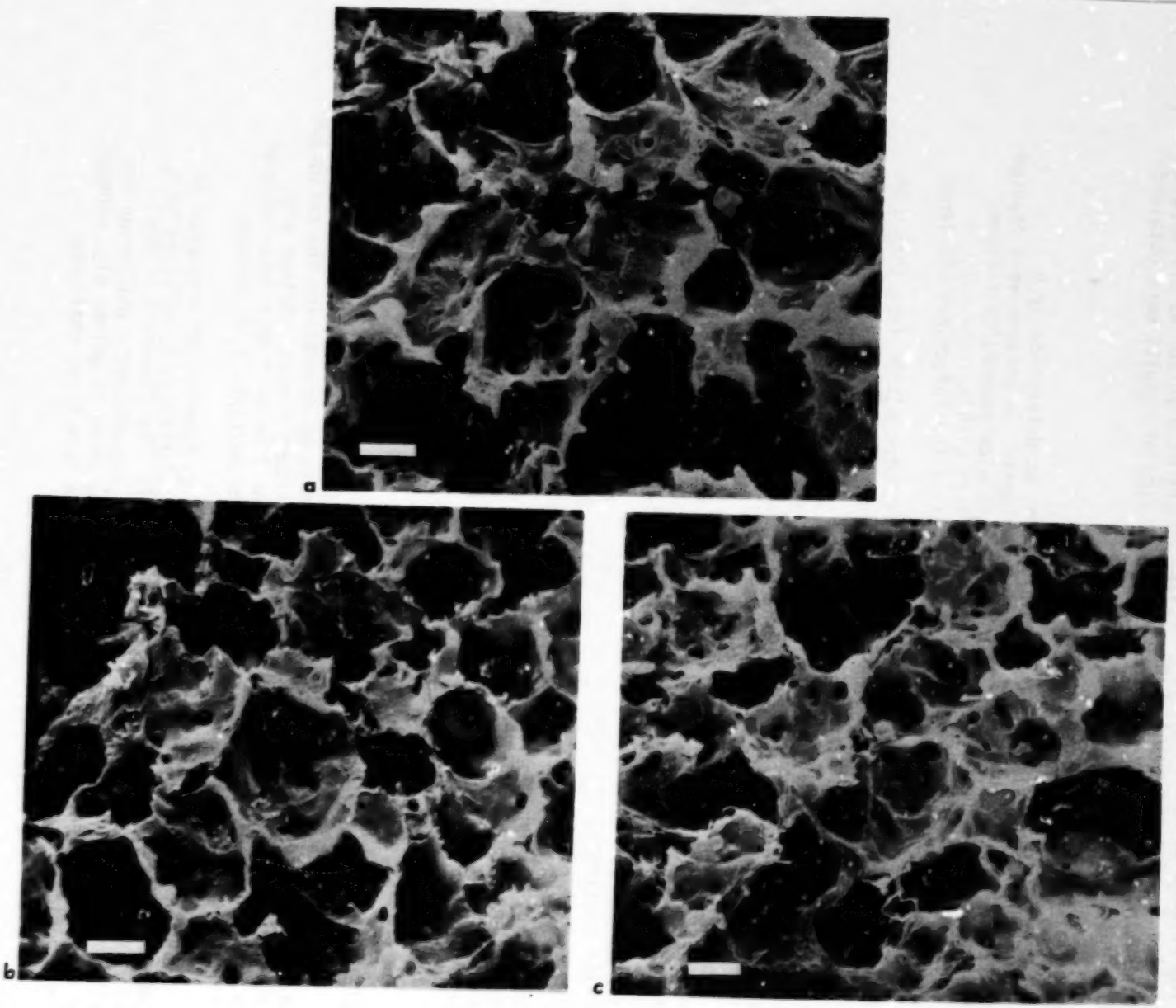

Figure 6. Lungs of mice exposed to 7.4 mg/m^3 PAN 3 hr/day, 5 days/week for 2 weeks. (a) normal tissue in 6 week-old-mice exposed to air; (b) slight thickening of alveolar walls in 16 week-old-mice exposed to air; (c) pronounced thickening of alveolar walls in 16 week-old mice exposed to PAN (20 μm).

EFFECTS OF OZONE ON RESISTANCE TO CHRONIC RESPIRATORY INFECTION

The effects of ozone on the resistance to chronic mycobacterial infection were studied in mice and guinea pigs. Although CF_1 mice served as the experimental host in the majority of the experiments, similar results were obtained when CDF or C57BL/6 mice were used.

Mycobacterial Titers in Lungs

The changes in resistance to the infection, as determined by *M. tuberculosis* titers in lungs and, in some experiments, in spleens were studied in mice exposed to ozone and compared to those in mice exposed to filtered air. In all experiments mice were challenged first with *M. tuberculosis* aerosol and exposed to various ozone regimens after the infectious challenge.

A single 3 hr exposure to 2.9 mg/m^3 O_3 initiated within less than 3 hr or 7 days after the challenge with *M. tuberculosis* aerosol resulted in bacterial lung titers that did not differ from those seen in mice exposed to air. Similarily no marked changes in titers were seen in lungs or spleens in mice challenged with *M. tuberculosis* aerosol and within $\leq$3 hr, 7 days or 14 days exposed 3 hr/day for up to 7 weeks to 0.98 mg/m^3 O_3.

Multiple daily exposures to 1.96 mg/m^3 O_3, 3 hr/day, 5 days/week for up to 8 weeks initiated at 7 and 14 days after the challenge with *M. tuberculosis* aerosol resulted in increased bacterial titers in lungs when compared to those of mice exposed to filtered air. In all groups, irrespective whether or not exposed to ozone, a lag in bacterial growth was seen during the first week after the challenge. In lungs of mice exposed to air a peak *M. tuberculosis* titer was attained at 4 weeks after the challenge and a slow decrease was seen thereafter. A similar initial lag and growth pattern was seen in mice exposed to ozone and a peak bacterial titer in lungs also appeared at 4 weeks after the challenge. However, the titers were markedly higher than those seen in lungs of mice exposed to filtered air. A decrease in titers occurred during the ensuing 4-week period with the titer being persistently higher than in mice exposed to air (Figure 7). At most time intervals the differences in lung titers were significant ($p \leq 0.05$).

Immune Response

The effects of ozone on cell mediated and humoral immune responses to tuberculosis infection were studied in guinea pigs by determining the cutaneous delayed hypersensitivity reaction and serum antibody titers. Guinea pigs were challenged with *M. tuberculosis* aerosol and 3 hr or 7 days later exposed to various concentrations of ozone for single or multiple 3 hr periods.

Continuous sensitivity to PPD, expressed as diameter of erythemas in guinea pigs infected with *M. tuberculosis*, was significantly affected by a single 3 hr exposure to 2.9 mg/m^3 O_3 (Figure 8). The diameters of the erythemas in guinea pigs exposed to ozone were significantly smaller during the 4 to 7 week period, but did not differ from those in guinea pigs exposed to air during the 8 to 18 week period after the infectious challenge.

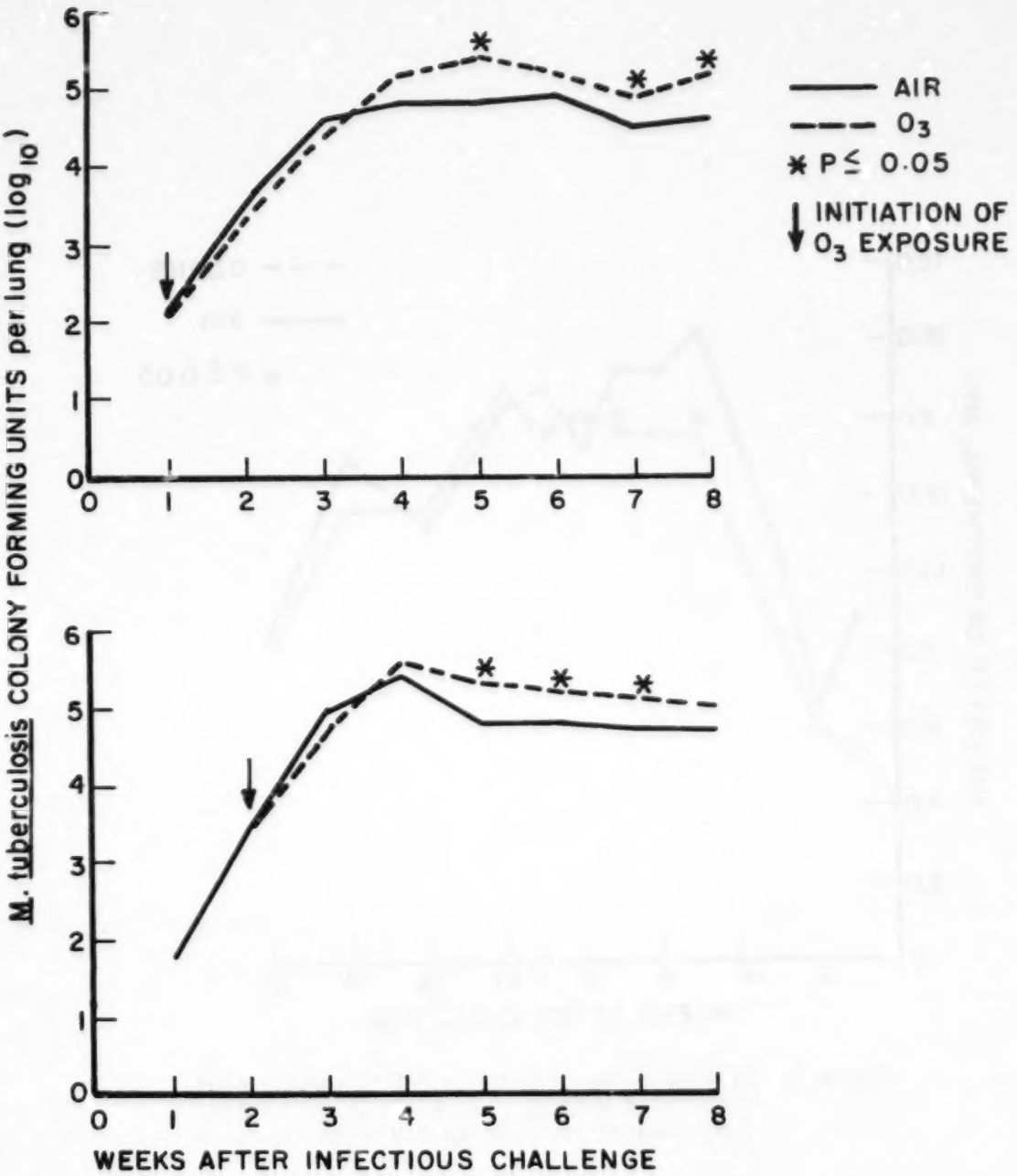

Figure 7. Titers of *M. tuberculosis* in lungs of mice exposed to 1.96 mg/m^3 O_3 3 hr/day, 5 days/week for 6 to 7 weeks.

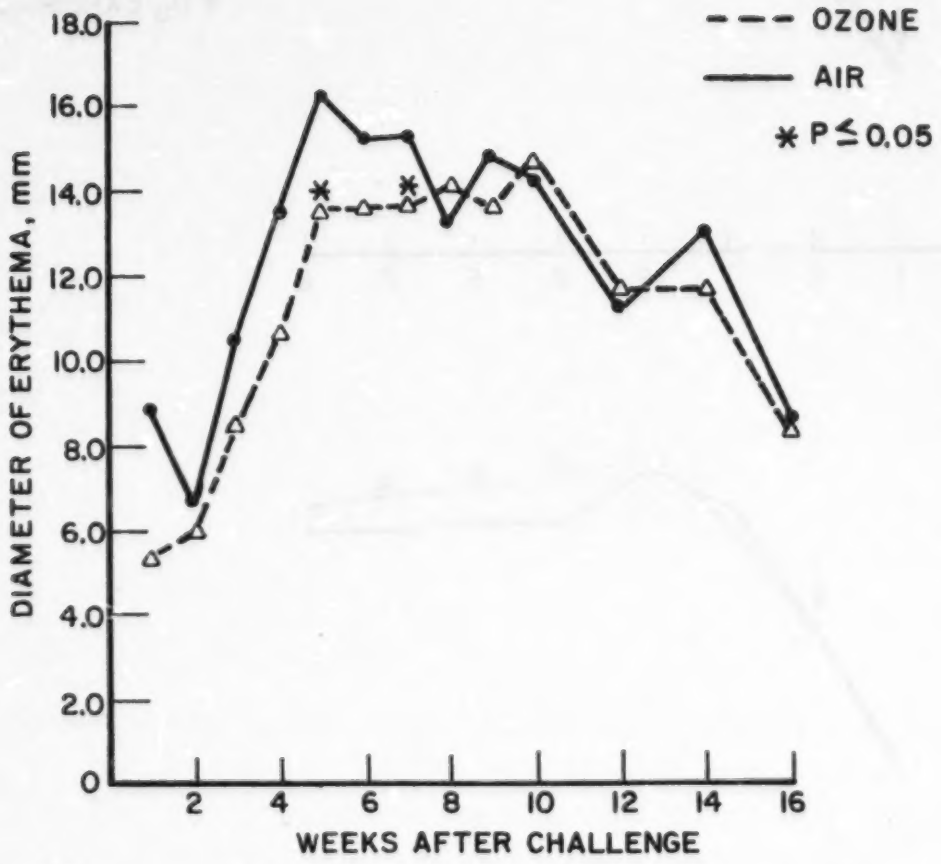

Figure 8. Cutaneous sensitivity to PPD in guinea pigs challenged with *M. tuberculosis* and within 3 hr exposed for 3 hr to 2.9 mg/m^3 O_3.

The peak response (i.e., the largest diameter) in guinea pigs exposed to ozone occurred at 10 weeks instead of 5 weeks as in those exposed to air. The hemagglutination antibody titers determined in the same guinea pigs exposed to ozone ranged from 16 to 128 over a 14 week observation period. The titers did not differ from those seen in animals exposed to filtered air.

Exposure to 0.98 mg/m^3 O_3, 3 hr/day for 5 days initiated within 3 hr after the challenge with *M. tuberculosis* aerosol had no effect on the hypersensitivity response in guinea pigs. The diameters of erythemas measured at weekly intervals during a 36 week observation period did not differ from those seen in guinea pigs exposed to filtered air.

The hemagglutination antibody titers were determined at approximately 3 week intervals during the 15 weeks. The titers were markedly higher in guinea pigs exposed to ozone than in those exposed to air during the initial 12 weeks (Figure 9). The peak antibody titer appeared to be attained at 9 weeks after the challenge and the titers decreased slowly during the remaining 7-week period. In guinea pigs exposed to air, the peak antibody titer appeared at 15 weeks after the challenge.

In summary, single 3-hr exposures to 2.9 mg/m^3 O_3 caused a suppression of delayed hypersensitivity in guinea pigs, especially during the peak response at 5 and 6 weeks after infectious challenge with *M. tuberculosis* aerosol. Serum antibody titers were not altered. Daily 3-hr exposures to 0.98 mg/m^3 O_3 for 5 days did not suppress delayed hypersensitivity, but enhanced the serum antibody titer. Since the host response to tuberculosis involves primarily cell-mediated immunity it is reasonable to suggest that a suppression of the delayed hypersensitivity response, which is associated with cell-mediated immunity, enhanced the local lung infection in mice exposed to ozone. Although not conclusively demonstrated, a suppression of delayed hypersensitivity appeared to be present in most of the guinea pigs exposed to ozone.

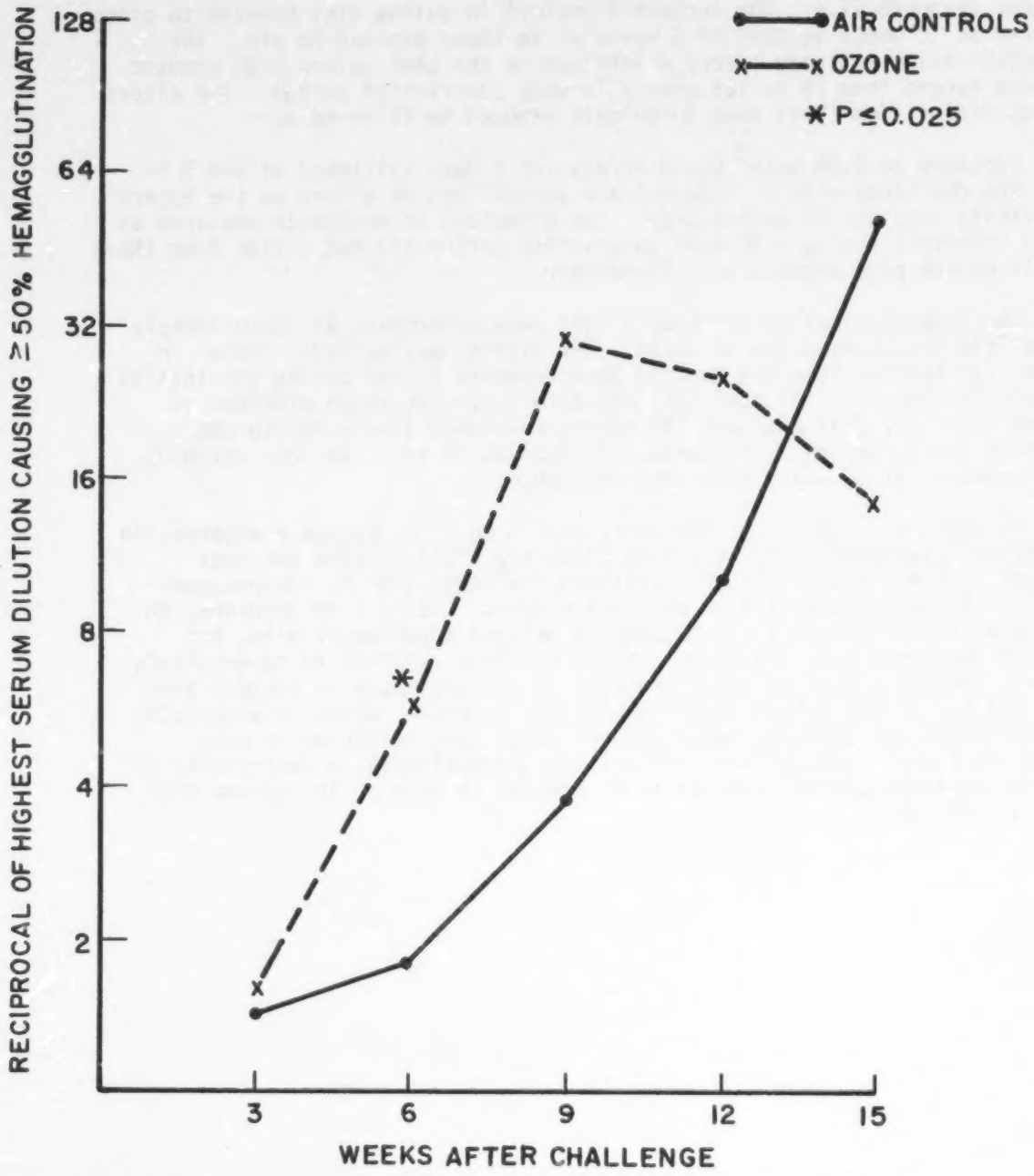

Figure 9. Serum hemagglutination antibody titers in guinea pigs challenged with *M. tuberculosis* and within 3 hr exposed to 0.98 mg/m^3 O_3 3 hr/day for 5 days.

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